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Tobacco Smoke and Toxicology

PHYSICAL AND CHEMICAL CHARACTERISTICS OF TOBACCO SMOKE

Tobacco smoke is a complex mixture of toxicants and the chemical properties change—rapidly in some cases—as smoke ages. Toxicants measured at one point in time may not be what the smoker actually experiences. It is estimated that there are more than 2,000 chemical constituents of tobacco. Almost twice that number results when tobacco is burned incompletely during smoking. Three kinds of smoke can be described, each differing in terms of toxicant concentration, size of particles, effects of temperature, and a host of other characteristics. Mainstream smoke (MS) is what emerges from the "mouth" or butt end of a puffed cigarette. Sidestream smoke (SS) is what arises from the lit end of a cigarette, mostly between puffs. Environmental tobacco smoke (ETS), smoke present in air, consists of exhaled mainstream smoke and sidestream smoke.

Smoking machines are used to analyze mainstream smoke. A set of parameters has been agreed on by various international organizations. These parameters are 35-cm^3 puff volume, 2-second puff duration, once per minute puff interval, and smoking to a butt length of 23 mm for nonfiltered or 3 mm above the filter overlap for filter-tipped cigarettes. The "yields" of toxicants in the MS are frequently reported by the standard-setting organization. The two most well known organizations are the U.S. Federal Trade Commission (FTC) and the International Organization of Standardization (ISO). The controversy regarding the standard parameters and reporting of values is covered in other chapters of this report.

Mainstream smoke is pulled through the mouth end of the cigarette and then through a "Cambridge filter pad". Aerosol particles in the smoke larger than 1 μm in diameter are trapped with 99% efficiency. The material is referred to as cigarette smoke condensate or total particulate matter (TPM). "Tar" is the weight of TPM minus nicotine and water. The material that passes through the filter pad is the gas or vapor phase of cigarette smoke. In general, the vapor phase consists predominantly of compounds with a molecular weight <60 and the particulate phase consists of compounds with a molecular weight >200.

The yields of MS increase with successive puffs as the cigarette is machine-smoked due to the decrease in filtration provided by the cigarette rod itself. However, smoking behavior studies coupled with yield measurements suggest that yields remain consistent from first puff to last when assessed under real-life smoking conditions.

When tobacco is heated, moisture and volatile material are distilled, and pyrolysis leads to the generation of volatile gases and the residual, carbonized char. Char reacts with oxygen in the air during puffing and smoldering, producing volatile gases (carbon dioxide, carbon monoxide, and water) and the inorganic material known as ash.

The highest temperature reached during the burning of tobacco is approximately 800°C in the center of the burning zone during smolder. During puff, a solid-phase temperature of approximately 910°C is reached at the burning zone periphery, while the gas temperatures are lower. They vary between 600 and 700°C as the puff progresses. After the puff ends, solid-phase temperatures rapidly cool to approximately 600°C. This greatly influences particle formation, particle size, and toxicant formation. These temperatures contrast with that achieved with a newly marketed cigarette-like device, Eclipse™, that combusts differently than conventional cigarettes and aerosolizes nicotine and glycerin.

The chemical nature of MS changes as smoke ages. The burning zone generates a highly concentrated vapor that is drawn down the cigarette to form mainstream smoke. The vapor cools quickly (in milliseconds) due to diluting air. Less volatile compounds quickly condense, mostly in airborne state. A combination of physical size and concentration affects both thermal and mechanical properties, which influence the number of particles in smoke. Droplets of less than about 0.1 μm will attach to the tobacco through which they pass or to other particles, which continue on into MS. Particles with sizes around 1 μm are "filtered" out by depositing onto the tobacco surface.

MS is a highly concentrated aerosol mixture. Smoke particles are liquid, consisting of approximately 20% water by volume. The particles vary from less than 0.1 to 1.0-μm diameter. The small size and high concentration promote rapid coagulation, leading to decreased concentration and increased size of the resulting particles within less than a second. The size of particles also increases due to absorption of water, which is relevant for human smoking because of the high relative humidity of the human respiratory tract.

Sidestream smoke particles are smaller than MS particles initially. However, the aging of SS over a few minutes leads to an increase in particle size of ETS due to coagulation of particles and removal of smaller particles that attach to surfaces in the environment. Particle size in smoke is important, because it influences where within the respiratory tract a toxicant is deposited. Smaller particles, in general, deposit further down into the lungs.

Inhaled particles of the size found in tobacco smoke would be predicted to deposit mainly in the alveolar region of the lung. However, cigarette smoke-induced tumors are more prevalent in the bronchial region, suggesting that smoke particles deposit higher up in the respiratory tract than would be predicted from the initial particle size. (Recent increases of adenocarcinomas in lower airways of smokers are hypothesized to be due to so-called smoking compensation of low-yield products. Smokers of these products inhale more deeply to increase their nicotine dose.) Mucociliary clearance of inhaled particles up the respiratory tract may also increase the dose of particles to the upper airways. More importantly, the cloud-like nature of MS (see below) and the increased size of smoke particles on aging are responsible for this finding. Specific factors influencing the site of deposition in airways include coagulation of fresh smoke particles, absorption of water in the humid respiratory tract, human breathing patterns, aerodynamic interactions between nearby particles, electrostatic charge, and vapor deposition on airway walls (Dendo et al., 1998). Theoretical models of particle deposition predict that MS particles would have approximately 20% deposition in the respiratory tract. Measurements in humans suggest that deposition is actually much higher, from 50 to 95% (Phalen, 2000).

The explanation for this high deposition rate is that cigarette smoke is so dense, that it acts as a cloud. Clouds are high concentrations of aerosol particles surrounded by relatively clean air. They behave as entities that are much larger than the individual components. In depositing, the cloud behaves as if it were a much larger particle, with an aerodynamic diameter of

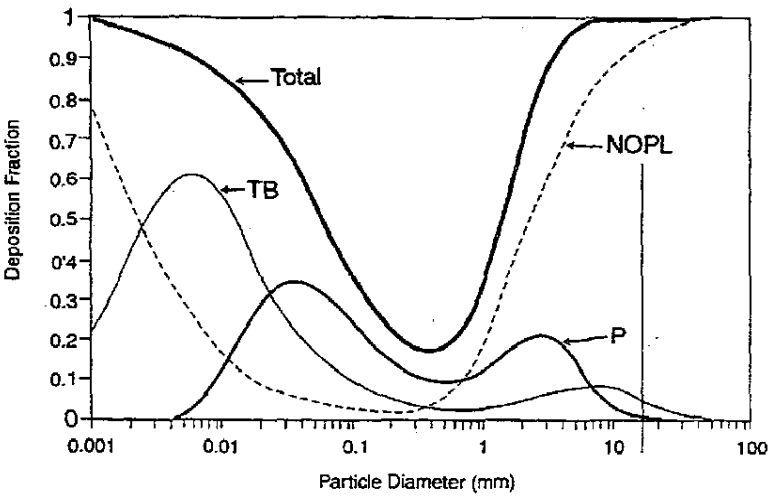

FIGURE 10-1 Aerosol deposition curves.

NOTE: applies to the Reference Man; NOPL = nasal oral pharyngeal laryngeal regions; TB = tracheobronchial region. SOURCE: Adapted from National Council on Radiation Protection and Measurements report #125. 1997. with permission form the National Council on Radiation Protection and Measurements.

approximately 6 or 7 μm. This particle size (see Figure 10-1) results in high total deposition in the respiratory tract, with especially high deposition in the tracheobronchial region.

Deposition in the respiratory tract is also influenced by the size of the person. Smaller individuals have greater tracheobronchial and less pulmonary deposition. Smaller individuals also have greater minute ventilation normalized for body weight, which is especially important for SS. This is hypothesized to be one reason infants might be more vulnerable to ETS. Importantly the small airway diameter of infants leads to high disposition in the upper airways, where it can be an irritant (Phalen, 2000).

Whole MS consists (by weight) of mostly air (nitrogen N_2, oxygen O_2, argon). The vapor phase constitutes approximately 20% by weight of the smoke, with the particulate phase accounting for approximately 5%.

The majority of cigarettes sold today have filters to remove portions of the smoke. Cellulose acetate filters are used almost exclusively in the United States, whereas charcoal filters are popular in Japan (Norman, 1999). Cellulose acetate filters remove some of the particulate phase of the smoke but have little influence on the vapor phase. The efficiency of particle removal depends on particle size and is minimal at the number-average particle size found in cigarette smoke (about 0.3 μm diameter). In general, cellulose acetate or paper filters remove tar and nicotine particles in this size range with an efficiency of 40–50%. Charcoal filters influence the retention of vapor-phase components and are made by adding up to 60 mg of activated charcoal in a segment of the cellulose acetate filter. Factors that influence the retention of vapor-phase compounds include the amount of charcoal used, the activity of the charcoal (based on surface area and pore volume), and the smoke velocity through the segment. Low-molecular-weight compounds with low boiling points (less than 150°C), which are not retained by the cellulose acetate filters, are partially removed by the charcoal filters. Removal efficiencies vary with the compound but are reported to be from 30 to 90%. As an example, benzene may be removed at an efficiency of 67%. Vented filters are designed to improve filter efficiency by decreased smoke

flow through and increased residence time in the filter. However, vent holes can readily be covered by the fingers of the smoker, who may be inclined to do this in order to get the maximum amount of nicotine during smoking.

Under similar smoking conditions, filtered cigarettes will have lower MS yield relative to nonfiltered analogues. SS yields will not vary much, since they are reflective of tobacco weight burned during smolder. In general, more tobacco is consumed during smolder than during puffing. However, SS generally contains more alkaline and neutral compounds. SS smoke contains less or equal amounts of acids, phenols, and phytosterols than MS. Differences are due to temperature and mechanisms of chemical transfer (release) from the unburned tobacco. The approximate chemical composition of MS is given in Table 10-1. The relative concentration of specific constituents in MS versus SS tobacco smoke is shown in Table 10-2.

Table 10-1 Approximate Chemical Composition of Whole Mainstream Smoke

Constituent	% by Weight	
Air		
N_2	62	75.9
O_2	13	
Ar	0.9	
Vapor Phase		
Water	1.3	19.6
CO_2	12.5	
CO	4	
H_2	0.1	
CH_4	0.3	
Hydrocarbons	0.6	
Aldehydes	0.3	
Ketones	0.2	
Nitriles	0.1	
Heterocyclics	0.03	
Methanol	0.03	
Organic acids	0.02	
Esters	0.01	
Other compounds	0.1	
ParticulatePhase		
Water	0.8	4.5
Alkanes	0.2	
Terpenoids	0.2	
Phenols	0.2	
Esters	0.2	
Nicotine	0.3	
Other alkaloids	0.1	
Alcohols	0.3	
Carbonyls	0.5	
Organic acids	0.6	
Leaf pigments	0.2	
Other compounds	0.9	

NOTE: Ar = Argon; CH_4 = methane; CO – carbon monoxide; CO_2 = carbon dioxide; H_2 = hydrogen; N_2 = nitrogen; O_2 = oxygen.

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The pH of cigarette smoke influences the degree of protonation of the active addictive chemical, nicotine. The free-base form of nicotine is favored at a higher pH (more basic) and is more rapidly absorbed into the bloodstream than the mono- or diprotonated salt forms of nicotine that exist at lower pH (more acidic). Tobacco blends with a high sugar content produce a more acidic smoke; a basic cigarette smoke can be achieved by addition of ammonia.

Table 10-2 Some Typical SS/MS Yield Ratios for Plain Cigarettes

Substance	**MS Yield**	**SS/MS**
Small Molecules		
Carbonyl sulfide	18—42 µg	0.03—0.1
HCN	160—500 µg	0.06—0.5
CO	10—23 mg	2.5—4.7
Hydrazine	20—43 µg	3
Methane	600—1000 µg	3.1—4.8
Acetylene	20—40 µg	0.8—2.5
Nitrogen oxides	100—600 µg	4—13
CO_2	20—50 mg	8—11
H_2O (gas phase)	3—14 mg	24—30
NH_3	50—130 µg	40—170
N_2 (generated)	<10 µg	>270
Neutral Heteroatom Organics		
Acetonitrile	50—130 µg	40—170
Benzonitrile	5—6 µg	7—10
Acetamide	70—100 µg	0.8—1.7
Methyl chloride	150—600 µg	1.7—3.3
Aldehydes, Ketones, Alcohols		
Acetaldehyde	0.5—1.2 mg	1.4
Propionaldehyde	175—250 µg	2.4—2.8
Acetone	100—250 µg	2—5
Acrolein	60—100 µg	8—15
2-Butanone	—30 µg	2.9—4.3
2-Furaldehyde	15—43 µg	4.9—7.4
Furfuryl alcohol	18—65 µg	3.0—4.8
Cyclotene[a]	3—5 µg	6—10
Pyranone[b]	13—150 µg	0.1—1.2
Phytosterols		
ß-Sitosterol	59 µg	0.5
Campesterol	43 µg	0.6
Cholesterol	22 µg	0.9

Phenols		
Phenol	60—140 µg	1.6—3.0
Cresols (o-,m-,p-)	11—37 µg	1.0—1.4
Catechol	100—360 µg	0.6—0.9
Hydroquinone	110—300 µg	0.7—1.0
Acids		
Formic acid	210—490 µg	1.4—1.6
Acetic acid	270—810 µg	1.9—3.9
3-Methylvaleric acid	20—60 µg	0.8—1.5
Lactic acid	60—170 µg	0.5—0.7
Benzoic acid	14—28 µg	0.7—1.0
Phenylacetic acid	11—38 µg	0.6—0.8
Succinic acid	70—140 µg	0.4—0.6
Glycolic acid	40—130 µg	0.6—1.0
Amines, Pyridines, Alkaloids		
Methylamine	12—29 µg	4.2—6.4
n-Propylamine	1.6—3.4 µg	2.8—3.8
n-Butylamine	0.5—1.5 µg	2.2—4.0
Aniline	360 ng	30
Pyridine	16—46 µg	6.5—20
3-Ethenylpyridine	11—30 µg	20—40
Methylpyrazine	2—5 µg	3—4
Pyrrole	16—23 µg	9—14
Nicotine	0.8—2.3 mg	2.6—3.3
Myomine	13—33 µg	4.0—7.5
Nicotyrine	4—40 µg	5—14
Anatabine	2—20 µg	0.1—0.5
2,3'-Bipyridyl	16—22 µg	2—3
Aza-arenes		
Quinoline	0.5—2.0 µg	8—11
Isoquinoline	1.6—2.0 µg	2.5—5
Benzo[h]quinoline	10 ng	10
Indole	16—38 µg	2.1—3.4
Hydrocarbons		
Isoprene	330—1100 µg	13—19
Benzene	36—68 µg	5—10
Toluene	100—200 µg	6—8
Limonene	15—50 µg	4—12
Neophytadiene	66—230 µg	1—2

Polynuclear Aromatic Hydrocarbons		
Naphthalene	2.6 µg	17
Pyrene	45—140 µg	2—11
Benzo[a]pyrene	9—40 µg	2—20
Anthracene	24 ng	30
Phenanthrene	77 ng	2—30
Fluoranthene	60—150 ng	11
Nitrosamines[c]		
N-Nitrosodimethylamine	10—40 ng	10—50
N-Nitrosodiethylamine	nd—25 ng	3—35
N-Nitrosopyrrolidine	6—30 ng	6—30
N-Nitrosodiethanolamine	0—70 ng	1.2
N'-Nitrosonomicotine	0.2—3 µg	0.5—3
NNK[d]	0.1—1 µg	1—4
N'-Nitrosoanatabine	0.3—5 µg	0.3—1
Inorganic Constituents		
Cadmium	100 ng	4—7
Nickel	20—80 ng	0.2—30
Zinc	60 ng	0.2—7

NOTE: CO = carbon monoxide; CO_2 – carbon dioxide; HCN = hydrogen cyanide; H_2O = water; N_2 = nitrogen; nd = not detected; NH_3 = ammonia; NNK = nicotine-derived nitroketone.

[a] Hydroxy-3-methyl-2-cyclopentanone.

[b] 5m6-Dihydro-3,5-dihydroxy-2-methyl-4H-pyran-4-one.

[c] Much of the date in the literature on the smoke levels of volatile and tobacco-specific nitrosamines may be in error, due to artifact formation on the Cambridge pad part of the smoke collection procedure (Caldwell and Conner, 1990).

[d] Nitrosonomicotine ketone or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

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TOXICITY OF TOBACCO SMOKE

The health effects of tobacco smoke in humans are well known from both clinical and epidemiological studies; such information is summarized in later chapters of this report. Animal studies of the toxicity of tobacco smoke are reviewed in the present section. The purpose of this review of animal models of tobacco smoke toxicity is to determine the potential usefulness of such models for assessing the toxicity of new and existing products that claim to reduce harm from tobacco use. The possibilities and the limits of using animals to test for toxic effects related to tobacco use are discussed. The major adverse tobacco smoke-induced health effects that re-

quire evaluation because of their prominence in humans are pulmonary inflammation, induction of lung cancer, chronic obstructive pulmonary disease (COPD), cardiovascular disease, reproductive and developmental effects, and the suppression of the immune system.

In Vitro Toxicity Tests

Toxicity tests that can be performed on cell systems in vitro have the advantage of being done rapidly and with relatively low cost. Such tests can be used to screen for general toxic properties of a chemical or a chemical mixture, such as the cytotoxicity of the material or its ability to alter the genetic material, DNA. The cytotoxicity of a compound can be predictive of its ability to induce inflammation; the genotoxicity of a compound suggests its potential to induce cancer. The limitation of such tests is that the results are based on the response of single cell types and do not include the influence of the whole-body system on the response. Nevertheless, such tests can be valuable in excluding products from further development if they either are extremely cytotoxic or have a high potential for producing mutations in DNA. Cellular screening assays should include benchmark materials of known cytotoxic or genotoxic potential (based on both in vitro and in vivo studies) for comparison to the test material and to allow better interpretation of the results.

Cytotoxicity Tests

Cytotoxicity tests (Balls and Clothier, 1991) are based on either primary cultures or established cell lines from the target organ of interest. Dye exclusion or the release of cytoplasmic enzymes is used to measure damage to cell membranes. In dye exclusion tests, the ability of cells to exclude extracellular dyes such as trypan blue or neutral red is measured. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) is commonly measured as an indicator of cell membrane damage. For specific purposes, the release of other enzymes, such as the hydrolytic enzymes of pulmonary macrophages, can be useful. The exclusion of the dye, neutral red, and the release of LDH have been recently used to compare the cytotoxicity of smoke condensates from standard cigarettes and a new tobacco-related PREP for which harm reduction was claimed (Eclipse Expert Panel, 2000)

Genotoxicity Tests

Several in vitro tests designed to assay for the mutagenic potential of a chemical or mixture, were recently reviewed in an International Workshop on Genotoxicity Test Procedures (Lovell et al., 2000). Perhaps the most commonly used screening tool is the *Salmonella typhimurium* bacterial mutagenicity assay or Ames test (Ames et al., 1987). In addition to assays for point mutations, there are assays of clastogenic DNA damage as indicated by chromosomal aberrations, sister chromatid exchanges, micronuclei formation (Hayashi et al., 2000), or single strand breaks via the Comet assay (Tice et al., 2000). Assays for specific chemical adducts to DNA can be used as a measure of dosimetry and, in a few cases, as predictors of adverse effects (Phillips et al., 2000). Measures of oxidized bases in DNA can be used to assay for oxidative stress (Cadet et al., 1998). In recent comparative potency studies comparing the genotoxicity of smoke condensates from standard and new tobacco-related PREP, a battery of in vitro assays included the Ames test, SCEs, and chromosomal aberrations (Eclipse Expert Panel, 2000).

In Vivo Toxicity Tests

Animals do not smoke cigarettes in the same manner as humans, and much effort has been expended in the past in animal studies to mimic human exposures to intermittent puffs of smoke. It is impractical to replicate all of the parameters of human smoking in animals. One problem is that rodents tend to hold their breath during puffs of irritating tobacco smoke and thus avoid receiving a high dose of smoke (Kendrick et al., 1976). Studies were conducted comparing three modes of exposure of rats to cigarette smoke: nose-only intermittent, nose-only continuous, and whole-body continuous (Mauderly et al., 1989; Chen et al., 1995). Plasma nicotine was higher by a factor of 3 in whole-body exposed rats compared to nose-only exposed rats. This suggests that dermal absorption and grooming as well as inhalation contributed to the dose of nicotine received in rats exposed in the whole-body mode. Urinary cotinine was not higher in the whole-body exposed group compared to the nose-only intermittent exposure group but was higher by a factor of 1.5 compared to the nose-only continuous exposure group. This study demonstrated few significant differences in either smoke characteristics or biological effects among the three exposure modes. The biological effects thought to be related to chemical carcinogenesis (cell transformation, chromosomal damage, DNA adducts) and chronic lung disease (cell proliferation, inflammation, respiratory function) were similar for all groups. Whole-body exposures were less labor intensive and less stressful to the rats (based on body weights) and avoided the reduction in breathing known to occur during puff-by-puff exposures. Thus, whole-body exposures may be useful as a method to achieve dosing of tobacco smoke in small laboratory test animals.

ASSESSMENT OF POTENTIAL EXPOSURE REDUCTION PRODUCTS

Lung Cancer

Animals have not proven to be good models for the type of lung tumors induced by cigarette smoke in humans. Rodents tend to develop peripherally arising lung adenomas rather than centrally arising bronchial tumors when exposed to chemicals. Exposure of animals to tobacco smoke has not often produced an excess of lung tumors of any type. In 1986, the International Agency for Research on Cancer (IARC, 1986) critically reviewed animal studies on tobacco smoke; out of four rat studies judged to be adequate for analysis, only one yielded unequivocal evidence for tobacco smoke as a respiratory tract carcinogen. One problem may be that rats build up carboxyhemoglobin faster than humans when exposed to the same level of carbon monoxide (CO) (Silbaugh and Horvath, 1982; Guerin et al., 1974). This results in their not being able to tolerate the level of exposure to cigarette smoke that humans can. Other factors undoubtedly contribute to this species difference in response to cigarette smoke. However, rodent models can be used to test the ability of tobacco products to cause alterations in DNA, and recent studies (discussed below) indicate that the A/J strain of mouse shows promise as a model for in vivo carcinogenesis induced by tobacco smoke.

Short-term exposure of rodents followed by analysis of DNA isolated from the lungs for DNA modifications such as oxidative damage or methylation, can determine the ability of the product to damage DNA in vivo. Aberrant methylation of DNA can be used as a marker for early stages of oncogenesis in both rats and humans (Nuovo et al., 1999; Belinsky et al., 1998; Swafford et al., 1997). Oxidative damage to DNA is used to monitor oxidative stress (Loft et al., 1998; Halliwell, 1998).

The A/J mouse strain, which is sensitive to induction of lung adenomas, has been used in a series of studies by Witschi to test for the carcinogenic potential of tobacco smoke and the effec-

tiveness of chemopreventive measures (Witschi et al., 1997a, 1999, 2000). The A/J mice exposed to 87 mg/m^3 of environmental tobacco smoke for 5 months and allowed to recover for 4 months had a statistically significant elevation in number of lung tumors (Witschi et al., 1997a). The same strain of mice exposed similarly to filtered and unfiltered tobacco smoke suggested that the particulate phase was not required for carcinogenicity (Witschi et al., 1997b). In a chemoprevention study, acetylsalicylic acid, an agent known to protect against nicotine-derived nitroketone (NNK)-induced tumors in the same strain of mice, had no protective effect against tobacco smoke (Witschi et al., 1999). A second chemoprevention study indicated that a diet containing myoinisitol–dexamethasone was effective in preventing tobacco smoke-induced lung tumors but that agents known to protect against NNK-or Polycyclic aromatic hydrocarbon (PAH)-induced tumors did not protect against tobacco smoke (Witschi et al., 2000).

The committee concludes that these studies indicate that removal from tobacco smoke of single classes of carcinogens, such as nitrosamines or PAHs, may not be protective against the induction of lung tumors by smoke. These studies also suggest that the A/J mouse, used in "stop–start" studies, shows promise as an animal model of value in screening for the potential of tobacco products to induce lung tumors. Future studies should determine if the model is robust enough to be repeated in other laboratories. In recent comparative potency studies for a newly developed tobacco-related PREP, the potency for smoke condensates to induce cancer was evaluated in 30-wk dermal tumor-promotion studies in mice (Eclipse Expert Panel, 2000). Such skin painting studies provide information for hazard identification.

Chronic Obstructive Pulmonary Disease

An early response to any inhaled toxicant is pulmonary inflammation, which if persistent may lead to more severe alterations in the structure and function of the lung. Animal models can readily be used to detect and quantitate the pulmonary inflammatory response to inhaled compounds or mixtures. Analysis of bronchoalveolar lavage (BAL) fluid for cellular and biochemical indicators of inflammation has become a common tool for quantitation of the pulmonary inflammatory response of rodents to inhaled toxicants (Henderson, 1989), including tobacco smoke (Mauderly et al., 1989; Subramaniam et al., 1996; Sjostrand and Rylander, 1997). The differential cell count and the functioning of cells obtained by the BAL technique can be used to classify the type of inflammatory response. The biochemical content of BAL fluid can be used to detect the release of various cytokines and alterations in the pulmonary surfactant.

Chronic obstructive pulmonary disease in the form of either emphysema, bronchitis, or both is a well-recognized sequela of cigarette smoking (Vial, 1986). Harm reduction strategies must take into account the degree to which this type of chronic lung disease is reduced in new products. Animals models to study the degree of COPD induced by the use of new products have been suggested by the work of March et al. (1999a,) F344 rats exposed to cigarette smoke over a 2-week period showed enhanced pulmonary epithelial cell replication and alterations in axial airway mucosubstances—changes consistent with the development of chronic bronchitis (March et al., 1999a). Both B6C3F1 mice and F344 rats exposed to cigarette smoke over a longer period (7–13 months) were found to develop morphological evidence of emphysema. Mice developed more pronounced signs of emphysema than rats, and the condition progressed with time in mice (March et al., 1999b). In earlier studies, rats exposed for 3 months to sidestream smoke were reported to have emphysematous changes in the lung (Escolar et al., 1995). Comparative potency studies for a newly developed tobacco-related PREP made use of 90-day inhalation studies in

hamsters and rats to test for inflammation as well as epithelial hyperplasia and metaplasia (Eclipse Expert Panel, 2000).

The committee concludes that these studies indicate there are animal models that show promise for use in screening for development of COPD-like symptoms from the inhalation of new or existing tobacco products.

Cardiovascular Disease

Cockerels and rabbits are two animal models that have been used to test for the cardiovascular effects of tobacco smoke. Penn and coworkers (1993, 1994) found that 16-week exposure of cockerels to (2–3 mg/m^3) increased the size of arteriosclerotic plaques in the aorta. Rabbits fed a cholesterol-rich diet and exposed for 10 weeks to 4 and 33 mg/m^3 showed a dose-dependent increase in the size of arteriosclerotic lesions in the aorta and pulmonary artery, as well as increased stickiness of platelets (Zhu et al., 1994). C57BL/6 mice have also been used to test for the effect of inhaled pollutants on the induction of atherosclerosis and enhancement of arterial fatty deposits in animals fed a high-fat diet (Lewis et al., 1999).

The committee concludes that such studies suggest animal models can be used to detect the potential for tobacco products to enhance the development of atherosclerosis.

Immune System Dysfunction

Smoking-related changes in the peripheral immune system in humans include elevated white blood cell counts, increased cytotoxic or suppressor and decreased inducer or helper T-cell numbers, slightly suppressed T-lymphocyte activity, significantly decreased natural killer (NK) cell activity, lowered circulating immunoglobulin titers (except for IgE, which is elevated), and increased susceptibility to infection. Similar effects have been observed in animals (McAllister-Sistilli et al., 1998; Sopori et al., 1994; Johnson et al., 1990), suggesting that animal models can be used to test for harm reduction to the immune system from use of new tobacco products or nicotine delivery devices. The major areas of interest are reduced host resistance to infections and tumors, suppression of the cellular and humoral immune system, and interference with macrophage cell function.

The effect of tobacco smoke on the immune system of humans and rodents depends on the duration and level of exposure. In general, short-term. low-level exposures do not affect the immune system or may be stimulatory, whereas longer-term exposures (6 months or more) or high levels of exposure are immunosuppressive.

The committee concludes that this finding indicates that long-term animal studies are required to evaluate adverse effects of tobacco products on the immune system.

Animals exposed to cigarette smoke for extended periods are more susceptible than naïve animals to tumor and infectious agent challenge. Mice exposed to cigarette smoke for 6 months or longer were more susceptible to intratracheally instilled Lewis lung or TKL5 tumor cells in terms of increased tumor growth, metastases, and early death than unexposed mice (Thomas et al., 1974b; Chalmers et al., 1975). Such changes are not observed in mice exposed to cigarette smoke for short periods of time (days). Chronic exposure of mice to cigarette smoke results in increased susceptibility to infectious agents such as murine sarcoma virus (Thomas et al., 1974a) and influenza virus (Mackenzie, 1976; Mackenzie and Flower, 1979).

Cellular immunity, as evaluated by phytohemagglutinin (PHA)-induced lymphoproliferative response or development of tumor-specific cytotoxic T cells, was initially increased but, on con-

tinued exposure, greatly decreased in mice exposed to cigarette smoke (Chalmers et al., 1975; Holt et al., 1975; Thomas et al., 1973). Lymphocytes from mice exposed chronically to tobacco smoke have a decreased response to the mitogen PHA and release factors that inhibit the cytotoxic activity of NK cells against tumor cells. T-cell suppression may be due to defective antigen processing or antibody production.

The humoral immune response is also suppressed by chronic exposure of mice to tobacco smoke, while acute exposures may stimulate the humoral response. The primary and secondary antibody production by lymphocytes in the lung, lymph nodes, and spleen of mice exposed to tobacco smoke for longer than 26 weeks and challenged by inoculation with sheep erythrocytes was decreased (Thomas et al., 1975).

Laboratory test animals can be used to demonstrate the ability of cigarette smoke to slow the mucociliary clearance of particles from the lung and to alter the function of pulmonary macrophages. This effect has been observed in humans (Cohen et al., 1979; Bohning et al., 1982) and animals (Mauderly et al., 1989). In the latter study, rats exposed for 8 weeks to cigarette smoke were exposed one time to cerium^{144} dioxide particles. Smoking increased the half-time of the short-term clearance of these particles by 63% and long-term clearance two-fold. The slowing of clearance of inhaled particles is an adverse health effect that should be considered in studies of tobacco product toxicity.

Alveolar macrophages from rats exposed to tobacco smoke for 6 months have a decreased ability to phagocytize *S. tuphylocoesus aureus* (Drath et al., 1979; Huber et al., 1980). Alveolar macrophages from rats exposed to tobacco smoke for 36 days or longer had an increased ability to release reactive oxygen species, a property dependent on the particulate faction of the smoke and not the gases. Clearance of *Pseudomonas aeruginosa* from rodents exposed to cigarette smoke for 36 weeks was slower than in controls (Holt, 1977). The decreased ability to clear particles, including pathogens, and the increased release of reactive oxygen species contribute to enhancement of inflammatory processes in the lung.

Reproductive and Developmental Effects

There are several reports that exposure of pregnant rats to either sidestream or mainstream tobacco smoke results in decreased birthweight of the pups (Witschi et al., 1994; Rajini et al., 1994; Leichter, 1989; Reznik and Marquard, 1980). Thus, animal models are capable of detecting tobacco smoke-induced growth retardation in utero. Rat models have also been used to demonstrate the adverse effects of maternal tobacco smoke exposure on lung maturation in utero (Lichtenbeld and Vidic, 1989), leading to an increase interstitial volume in the lung parenchyma. Another study demonstrated that postnatal rats exposed to tobacco smoke had reduced proliferation of their bronchiolar epithelial cells accompanied by increased levels of cytochrome P-450 enzymes (Ji et al., 1994). Studies in rodents have shown that rat pups exposed in utero to tobacco smoke have altered composition of pulmonary surfactant (Subramaniam et al., 1999).

The committee concludes that such studies indicate the potential usefulness of animal models to detect the interference of tobacco smoke products on airway epithelial cell development.

SYNERGISTIC EFFECTS WITH OTHER POLLUTANTS

Occupational exposures to materials such as asbestos or radon daughters have proven to have a synergistic interaction with tobacco smoke leading to greatly increased production of lung tumors in exposed workers who also smoke. Although rats are not good models for detecting the

induction of lung tumors from cigarette smoke alone, rats exposed to both cigarette smoke and plutonium oxide particles clearly revealed the synergistic effects of cigarette smoke on the induction of lung tumors in combined exposures (Finch et al., 1998). In the past, it has not been customary for regulatory agencies to require testing for synergistic effects of a new product with other substances.

The committee concludes that in the case of tobacco smoke, for which several synergisms are known, it would be wise to consider adding such a test to the standard regimen.

MOLECULAR BIOLOGY TESTING TOOLS

Recent advances in the area of molecular biology hold promise as future tools for toxicity screening. The technology for producing transgenic mice allows one to gain gene functions, while the development of knockout mice allows one to delete gene functions (Arbeit and Hirose, 1999). At present these tools are better suited for mechanistic studies than for screening purposes, but in the future, genetically altered animals may become the standards for testing for specific types of toxicity, just as genetically altered *Salmonella* bacteria have become standards for testing the mutagenic potential of xenobiotics.

DNA microarray chips, which consist of an array of thousands of specific cDNA sequences or genes on a chip, allow one to detect and quantitate messenger RNAs that are the transcription products of the specific cDNA samples on the surface of the chips. Thus, if one knows the specific genes that are up-regulated in association with the onset of a disease process, one could theoretically use the microarray technique to detect some of the earliest indicators that a disease process has begun (Nuwaysir et al., 1999). This type of tool should be invaluable in developing rapid screens for early indicators of developing disease in exposed animals (or for clinical purposes in humans) in contrast to the long time frame required to detect indicators of established disease in laboratory animals. The field is developing rapidly, and some microarrays designed to detect squamous cell carcinomas of the lung have already been reported (Wang et al., 2000). Future research will be required to determine which genes are up-regulated at different times during the progression of specific diseases so that microchip arrays can be designed as accurate and specific pre-clinical indicators of developing disease.

SMOKELESS TOBACCO TOXICITY

Smokeless tobacco products, traditionally, are differentiated into snuff and chewing tobacco; are not combusted but exert their effects by direct mucosal contact and consequent entry of toxicants into the bloodstream. Snuff is typically a finely ground tobacco product that is used orally or nasally. Snuff is manufactured in a variety of forms including moist, dry, and fine cut (Connolly et al., 1986). The oral tobacco form that is chewed or simply kept in the mouth is generally known as chewing tobacco. Chewing tobacco is also produced in different forms including plug, loose-leaf, and twist varieties (Connolly et al., 1986). (See Chapter 4 for a more in-depth description of smokeless tobacco products and use statistics.)

Smokeless tobacco products are composed primarily of fire or air-cured dark tobacco (Davis and Nielson, 1999). The tobacco then undergoes an extended aging process that involves heating or fermentation depending on the product. During production, various additives are used for the desired flavor and aroma. The chemical composition of smokeless tobacco products varies due to differences in tobacco composition and cut, additives, and curing or processing conditions. The differences are also found among countries for similar reasons. In Sweden, for example, moist

snuff (snus) has a lower level of tobacco-specific nitrosamines (TSNAs) due to processing differences and lack of fermentation compared to snuff in other countries. Generally, the TSNA levels in both U.S. and Swedish products have decreased over the last decade secondary to changes in processing methods, and TSNA levels in certain U.S. brands of snuff have approached the Swedish variety (Davis and Nielson; 1999, Ahlborn et al., 1997).

The exact chemical composition of smokeless tobacco, as in tobacco smoke, is difficult to assess. The main target of exposure in the smokeless tobacco user is the oral cavity and the upper aerodigestive tract. The lower digestive tract, however, is exposed at a certain level because of the swallowing of snuff particles within saliva. Common carcinogens found in smokeless tobacco include TSNAs, PAHs (especially benzo[a]pyrene) [BaP] and polonium -210. The concentration of TSNAs in snuff ranges from 5,280 to 141,000 parts per billion (ppb), which is hundreds to thousands times higher than that allowed in other consumer and food products (Connolly et al., 1986). TSNAs are thought to be important carcinogens in humans and have been proven to be potent carcinogens in animal studies. Among the TSNAs, NNK and N-nitrosonornicotine (NNN) have proven to be the most important carcinogens in smokeless tobacco in Europe and North America (Nilsson; 1998, Hoffmann et al., 1987). Hecht et al (1986) showed that oral exposure to NNK and NNN in rats caused lung tumors as well as oral tumors at the site of exposure. A snuff user (10 grams of snuff per day) is exposed to 24–46 μg of TSNAs per day compared to a pack per day smoker who is exposed to, on average, 16.2 μg of nitrosamines (Hoffmann et al., 1995). Snuff use also exposes the user to trace amounts of lead, cadmium, and selenium (Hoffmann et al., 1987).

Dark tobacco has a high level of nicotine, with 3.5–4.0% reported in certain brands (Davis and Nielson, 1999). In general the nicotine content per dose of smokeless tobacco product is higher than that of cigarettes, but the maximum serum nicotine levels are similar among all tobacco users (Benowitz, 1997). While there are interindividual differences in nicotine absorption and metabolism, nicotine is absorbed more gradually from smokeless tobacco than from smoking, and blood concentration persists over a longer period of time and even overnight (NIH Consensus Report, 1986). Overall, smokeless tobacco users are exposed to a greater amount of nicotine because of continued slow absorption of nicotine up to an hour after the tobacco is taken out of the mouth as well as the more alkaline pH, causing nicotine to be present in its unprotonated form contributing to better absorption (Benowitz et al., 1988; Hoffmann and Djordjevic, 1997).

Long-term smokeless tobacco use has been linked to oropharyngeal cancer (IARC, 1985: Mattson and Winn, 1989). The evidence has been more convincing for snuff than for chewing tobacco. As outlined in the 1986, National Institute of Health (NIH) Consensus Development Conference on the Health Implications of Smokeless Tobacco, case reports and controlled studies have consistently reported tumor growth in the location of smokeless tobacco contact with mucosa or skin, resulting in a risk of oral cancer up to 4.2 times that of non-tobacco users as reported in one influential study (Winn et al., 1981). The most common type of cancer attributable to smokeless tobacco is oral squamous cell carcinoma, but verrucous carcinoma has also been reported (Connolly et al., 1986). In contrast, recent epidemiological studies from Sweden have failed to confirm a link between Swedish snus use and cancer (Lewin et al., 1998; Schildt et al., 1998). In a large population-based study looking at risk factors for squamous cancer of the head and neck, Lewin et al. (1998) found no increased risk with the use of Swedish snuff.

Results of animal studies were initially mixed regarding the effects of oral, subcutaneous, or topical administration of smokeless tobacco in rodents (Pershagen, 1996; Main and Lecavalier, 1988). More recently, however, as noted in a review of the health hazards of moist snuff by Ahl-

born et al., (1997), there as been increased experimental support for the carcinogenicity of snuff. Surgically formed canals in the lips of rodents into which snuff and snuff extracts are placed have been used to more closely model the human practice of snuff dipping (Hoffmann and Djordjevic, 1997). Studies in rats and hamsters have shown a higher incidence of malignancy when there was exposure to both tobacco and herpes simplex type 1 virus or a cancer initiator (Connolly et al., 1986; Ahlborn et al., 1997). There has been inconclusive evidence linking snuff use to a variety of other cancers including prostate, pancreas, bladder, stomach, and kidney (Nilsson, 1998; Winn, 1997; IARC, 1985).

Smokeless Tobacco Research Recommendations

In terms of smokeless tobacco use as a strategy for harm reduction, more research is needed to investigate further some of the contradictory findings regarding the risk of oral cancer and cardiovascular disease. Swedish snus (lower TSNA and nicotine levels than American brands) should be evaluated as a possible harm reduction product since two recent epidemiological studies have suggested that it does not increase the risk of oral cancer and has favorable cardiovascular risk outcomes. More investigations into the cellular toxicity and genotoxic potential of smokeless tobacco extracts are needed. Smokeless tobacco may be a valid substitute for cigarette smoking but would pose specific risks in certain groups including pregnant women, those with inflammatory bowel disease, and those with established cardiovascular disease. Also, the population risks include concomitant smoking and adolescent use of smokeless tobacco as a gateway to cigarette smoking.

The same types of animal studies used to evaluate the toxicity of inhaled tobacco smoke could be done to evaluate the toxicity of smokeless tobacco products, such as Swedish snus or snuff, with a change in emphasis to the oral route of delivery. Based on known adverse health effects in humans, animal tests would be needed to evaluate the potential for smokeless tobacco to cause chronic inflammation or cancer in tissues of the oral cavity. Toxicokinetic studies would be required to determine other potential target organs for extracts of smokeless tobacco. Examples of in vitro studies include reports showing that smokeless tobacco causes pro-inflammatory changes in cultured endothelial cells (Furie et al., 2000) and activates the complement cascade, suggesting an inflammatory potential (Chang et al., 1998). Animal studies have been used to evaluate the potential of smokeless tobacco to induce oral cancer, as reviewed by Grasso and Mann (1998).

Thus, the committee concludes that pre-clinical toxicity testing should be of value for assessing the potential adverse health effects from use of smokeless tobacco.

GENERAL RESEARCH AGENDA AND RECOMMENDATIONS

Toxicology studies, both in vitro and in vivo, provide the opportunity to evaluate the potential harm reduction offered by potential reduced-exposure products (PREPs). The comparative potency of the PREP can be determined in a series of preclinical studies that include both the PREP and the standard tobacco product that can be replaced by the PREP, particularly tobacco-related PREPs (Figure 10-2). Such tests have recently been reported for a new cigarette-like product (Eclipse Expert Panel, 2000). The preclinical tests should include in vitro tests in both animal and human cells to determine the cytotoxicity and the genotoxicity of the tobacco product to which humans will be exposed. Such a test must include dose–response studies to determine the amount of the exposure material required to cause toxicity. Next, studies should be conducted

in vivo in the best animal models available to determine the comparative potency of the PREP versus the standard product in producing: (1) pulmonary inflammation, (2) COPD, (3) cardiovascular disease, (4) reproductive toxicology, and (5) pulmonary neoplasms. If these preclinical studies indicate that the PREP is less potent than the standard tobacco product, clinical studies should be conducted to determine acute toxic effects, the toxicokinetic properties, or the adverse effects of the PREP in humans. The determination of human health effects from chronic use of the new product can only be inferred from comparisons of the results of comparative tests in the old and the new product, but cannot be determined directly. Thus the testing approach will allow the rejection of risk reduction claims for products that are as toxic or more toxic in preclinical tests compared to products already on the market; however, only after long-term use of the product by many people could it be determined if the chronic toxicity of the new product is less than that of the standard product.

Figure 10-2 Pre-clinical studies for standard and new tobacco products

	In Vitro Studies		**In Vivo Studies**	**Clinical Studies In Humans**	**Epidemiological Studies**
	Animal Cells	Human Cells	Animals	Acute Effects	Chronic Effects
Standard Tobacco Product	✓	✓	✓	✓	✓
Modified Tobacco Product	✓	✓	✓	✓	?

Based on the above information, it is clear that preclinical toxicity testing in vitro and in vivo can be done to assess the potential health effects of new products before they are released for human use. It is beyond the scope of the committee's task to recommend the specific set of toxicity tests that should be done on new or existing tobacco products. The committee recommends that a panel of experts be convened to determine the specific set of toxicity tests and details of the testing regimens. Details to be considered include species and strains of test animals, duration of tests, end points of interest, dose–response considerations, biomarkers of dosimetry and response, and standard comparison products to be tested as positive and negative controls.

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